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Production of Tetracycline by *Streptomyces aureofaciens* in Synthetic Media

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The production of tetracycline[1] by fermentation was disclosed by Minieri *et al.* (1953). Details of this fermentation process in synthetic media of low chloride content are presented, and the culture isolation program which was carried out in conjunction with this study is discussed.

In this fermentation the composition of the medium and the strain of streptomycete are both important factors, since *Streptomyces aureofaciens* is capable of producing at least two antibiotic substances as pointed out by Backus *et al.* (1954). In the presence of chloride, which is incorporated within the chlortetracycline[2] molecule (Broschard *et al.*, 1949) and which is therefore essential for its production as disclosed by Petty and Matrishin (1950), the antibiotic formed was predominately chlortetracycline. In media low in chloride tetracycline predominates and the chlortetracycline fraction diminishes since 1 ppm of available chloride ion can produce at most 14 μg/ml chlortetracycline.

The simultaneous production of two or more antibiotics in a fermentation is well known, and the substances formed may be either closely related on a chemical or a biological basis or widely separated. Typical examples of closely related compounds produced simultaneously by the same organism are the penicillins (Clarke, 1949), streptomycins (Waksman, 1949), polymyxins (Brownlee, 1949), bacitracins (Newton and Abraham, 1950), cephalosporins (Crawford *et al.*, 1952), nisins (Berridge *et al.*, 1952), neomycins (Waksman, 1953), rhodomycins (Brockmann *et al.*, 1951), and candicidins (Lechevalier *et al.*, 1953). Compounds which differ in their structure and in their biological activity and are produced simultaneously by the same organism are illustrated by spinulosin, fumigatin, and gliotoxin (Menzel *et al.*, 1944); actidione, grisein, and streptomycin (Waksman *et al.*, 1948; Whiffen, 1948); rimocidin and oxytetracycline (Davisson *et al.*, 1951); fradicin and neomycin (Waksman, 1953); chlortetracycline and an antifungal compound (Duggar *et al.*, 1954); and fungicidin and an actidione-like antibiotic (Hazen and Brown, 1951).

The effect of medium and strain upon the concurrent production of these antibacterial and antifungal agents is well established. Calam and Levi (1944) found different types of penicillin produced in synthetic and natural media, and Smith and Bide (1944) established the phenylacetyl grouping as a necessary component of the medium for the production of penicillin G in contrast to penicillin F, which was formed in its absence. That different isolates derived from the same parent culture were capable of producing different types of penicillin was demonstrated by Calam and Levi (1944). Changes in medium were found by Whiffen (1948) to alter the ratio between streptomycin and actidione, and strain selection led to the sole production of either component. Perlman (1949) showed that substrains could be chosen which produced more of the desired streptomycin and less of mannosidostreptomycin than the parent. Mayer *et al.* (1951) reported on two antibacterial substances produced by *Actinomyces vinaceous* which were markedly influenced by the medium. Waksman (1953) pointed out that *Streptomyces fradiae* produced fradicin, neomycin A, and a subtilis factor along with neomycin, and that medium and strain influenced the proportion of the individual components of the neomycin complex. Backus *et al.* (1954) disclosed that different strains of *S. aureofaciens* possess the capacity to produce the antibiotics chlortetracycline and tetra-

[1] The trade-mark of American Cyanamid Company for the antibiotic tetracycline is Achromycin.

[2] The trade-mark of American Cyanamid Company for the antibiotic chlortetracycline is Aureomycin.

cycline under controlled conditions of fermentation, and Martin *et al.* (1955) demonstrated the effect of medium upon the production by *S. aureofaciens* of an antifungal agent and chlortetracycline as well as other antibacterial agents.

Experimental Methods

Culture Selection

The *S. aureofaciens* strain received from Minieri *et al.*, (1953) showed a heterogeneous morphology which was evident upon the comparison of single colonies on Waksman agar for the isolation of soil fungi (Waksman, 1922). Approximately 30 per cent of 100 isolates showed gross morphological variation as reflected in sharply reduced pigmentation. These pale yellow isolates were found either to be entirely void of detectable antibiotic activity in our base synthetic medium A or to exhibit weak response as shown in table 1.

Isolate T5, which was selected from the group of highest producers, gave consistent antibiotic production averaging 125 μg per ml and showed no gross morphological variation in 500 colonies. A spore suspension of culture T5 was irradiated with ultraviolet at an exposure sufficient to give 99.99 per cent kill, and 200 colonies were transplanted to agar tubes. A total of 19 isolates or 9.5 per cent showed various types of gross morphological variation. A spore suspension of culture T5 was also treated with X-ray;[3] an exposure of 100,000 Roentgen units was employed giving 99.5 per cent kill. A total of 340 colonies was picked and, of these, 149, or 40 per cent, were gross morphological variants. Of 45 natural selections and 135 X-ray-treated isolates, none showed a significant increase in antibiotic production; treatment with ultraviolet irradiation, however, resulted in 3 superior isolates in the series of 45 cultures tested. Culture UV8, which gave a 20 per cent increase in tetracycline production with average yields of 150 μg/ml, was selected for medium development studies; this culture has been deposited in the American Type Culture Collection, Washington, D. C. Isolates were screened in the base synthetic medium A as listed in table 2 and standard operating conditions were employed.

TABLE 1

Pigmentation and tetracycline yields of isolates in base synthetic medium A

Range of Yield	Pale Yellow Colonies	Deep Yellow Colonies	Total Colonies
μg/ml	%	%	%
0–24	94	12	42
25–49	3	16	12
50–74	3	4	5
75–99	0	20	12
100–124	0	36	22
>125	0	12	7

Twelve hundred and fifty derivatives of culture UV8 were studied. One hundred and sixty, or 23 per cent, of 700 ultraviolet-treated isolates and 190, or 38 per cent, of 500 X-ray-treated isolates were morphological variants. None of the 50 natural selections gave evidence of gross morphological change.

A total of 1030 ultraviolet- and X-ray-treated isolates of culture UV8 was screened for tetracycline production in various synthetic media as progress was made in medium development. There was a greater number of superior producers as well as a higher incidence of morphological types in the X-ray-treated

[3] Suspensions were X-rayed at the Physics Department of the Memorial Hospital, New York, through the kindness of Dr. H. Christine Reilly and Miss Elizabeth Focht.

TABLE 2

Effect of medium composition upon tetracycline yield in shaker flask fermentations with Streptomyces aureofaciens strain UV8

	Medium A	Medium B	Medium C	Medium D	Medium E
Sucrose (g/L)	30	30	40	40	40
$H_3C_6H_5O_7 \cdot H_2O$ (g/L)	0	0	11.5	12.8	12.8
$Na_3C_6H_5O_7 \cdot 5H_2O$ (g/L)	2.5	2.5	0	0	0
$(NH_4)_2SO_4$ (g/L)	3.3	3.3	0	6.0	6.0
$MgSO_4 \cdot 7H_2O$ (g/L)	0.25	0.25	0.25	0.25	0.25
KH_2PO_4 (g/L)	0.10	0.15	0.15	0.15	0.15
K_2HPO_4 (g/L)	0.10	0	0	0	0
$CaCO_3$ (g/L)	1.0	1.50	1.50	10.0	11.0
CH_3COOH (g/L)	0.40	0	0	0	0
NH_4OH, 28% (ml/L)	0	0	10.00	0	0
$MnSO_4 \cdot 4H_2O$ (g/L)	0.01	0.01	0.01	0.01	0.01
$ZnSO_4 \cdot 7H_2O$ (g/L)	0.04	0.04	0.04	0.04	0.04
$K_2Cr_2O_7$ (mg/L)	0.016	0.016	0.016	0.016	0.016
Avg Yield (μg/ml)	150	290	545	940	1350
Minimum Yield (μg/ml)	140	275	500	900	1250
Maximum Yield (μg/ml)	170	310	610	1000	1400

isolates than in either the ultraviolet-treated or the untreated isolates. Of 640 ultraviolet-treated cultures, only 3 gave significant increases in broth potency over the parent. Of these 3, culture UV184 was selected as a control for the testing of 390 derivatives from the X-ray treatment of culture UV8. Eight isolates were found which gave broth potencies within the range of those produced by culture UV184. The yields obtained with these selected isolates from culture UV8 in synthetic medium E ranged from an average of 1855 to 2150 μg/ml as shown in table 3.

Shaker Flask Fermentations

Standard procedures.

1. Preparation of cultures:
   a. Isolates are carried on Waksman agar for the isolation of soil fungi (Waksman, 1922) without adjustment of pH, and are incubated at 30 C for a period of 10 days to permit good sporulation. The formula for this agar follows (per L): glucose, 10.0 g; peptone, 5.0 g; KH_2PO_4, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; and agar, 20.0 g;
   b. Selected isolates are stored under oil or lyophilized.
2. Inoculum:
   a. Medium (per L): sucrose, 30.0 g; soybean meal, 5.0 g; $Na_3C_6H_5O_7 \cdot 5H_2O$, 1.0 g; $(NH_4)_2SO_4$, 3.3 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; KH_2PO_4, 0.10 g; K_2HPO_4, 0.10 g; $CaCO_3$, 1.00 g; $MnSO_4 \cdot 4H_2O$, 0.01 g; $ZnSO_4 \cdot 7H_2O$, 0.04 g; $K_2Cr_2O_7$, 0.016 mg; and CH_3COOH, 0.40 ml.
   b. Volume: 50 ml per 250-ml Erlenmeyer flask; 400 ml per 2000-ml Erlenmeyer flask.
   c. Shaker: reciprocating with 3½-in. stroke at 97 cycles per min.
   d. Temperature: 30 C.
   e. Inoculum: dry mycelial-spore transfer, liquid mycelial suspension, or lyophile.
   f. Period of incubation: 48 to 72 hr to give pH ranging between 4.5 and 5.0.

TABLE 3

Tetracycline yields obtained in synthetic medium E with selected isolates derived from Streptomyces aureofaciens strain UV8

Culture	Avg Yield	Range of Yield
	μg/ml	*μg/ml*
UV8	1350	1250–1400
UV8-UV184	2050	1910–2360
UV8-X154	2100	1960–2420
UV8-X169	2070	1845–2340
UV8-X237	2150	2040–2590
UV8-X314	1860	1855–1870
UV8-X348	1880	1790–1990
UV8-X353	1890	1860–1920
UV8-X380	1925	1750–2180
UV8-X381	1855	1810–1920

3. Fermentation:
   a. Medium E (per L): $H_3C_6H_5O_7 \cdot H_2O$, 12.8 g; sucrose, 40.0 g; $(NH_4)_2SO_4$, 6.0 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; KH_2PO_4, 0.15 g; $CaCO_3$, 11.00 g; $MnSO_4 \cdot 4H_2O$, 0.01 g; $ZnSO_4 \cdot 7H_2O$, 0.04 g; and $K_2Cr_2O_7$, 0.016 mg.
   b. Volume: 50 ml per 250-ml Erlenmeyer flask.
   c. Shaker: rotary at 200 rpm.
   d. Temperature: 30 C.
   e. Inoculum: 5 per cent mycelial transfer.
   f. Period of incubation: 4 to 6 days.
4. Preparation of sample for assay: Whole broth cultures are acidified to pH 2.0 to 2.5 with 5 N sulfuric acid and held for ½ hr before filtration through Whatman No. 4 paper. The paper disc method of assay with *Escherichia coli* strain ATCC no. 9637 is employed. Assays are run against a tetracycline standard.

Inoculum. The physiological state of the inoculum as indicated by pH, growth, and pigmentation was found to be a highly critical factor in this fermentation. Its effect was pronounced throughout the fermentation cycle, but was most evident in the early stages of growth. The optimum pH at transfer was found to range between 4.5 and 5.0 with significant reductions in harvest potencies when either physiologically older or younger inoculum was employed.

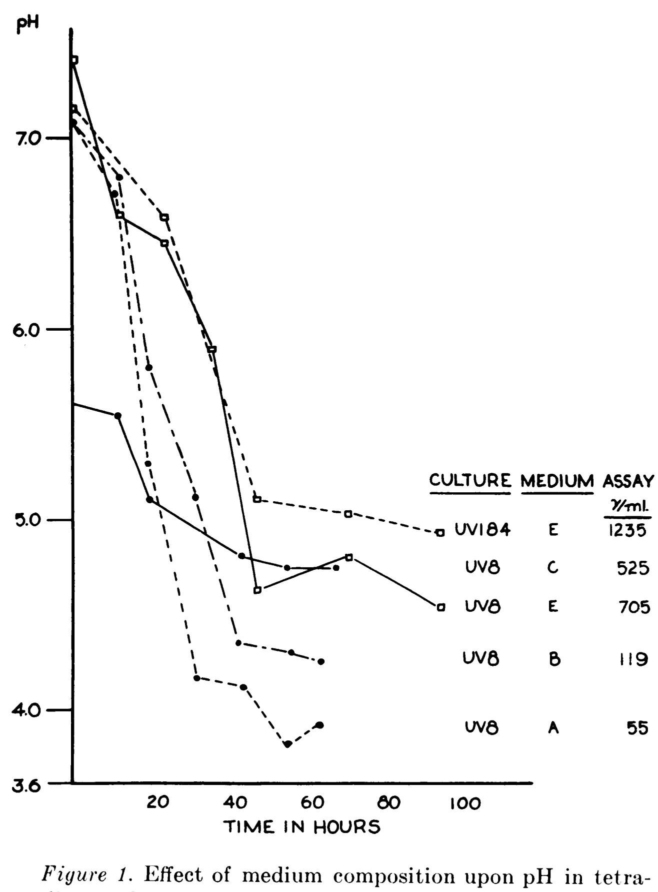

Figure 1. Effect of medium composition upon pH in tetracycline tank fermentations with *Streptomyces aureofaciens.*

Shaker flask fermentation. To limit production of chlortetracycline, all media contained less than 1 ppm chloride. The base synthetic medium A which we developed for this fermentation and in which culture T5 and UV8 were selected has the following formulation (per L): sucrose, 30 g; $Na_3C_6H_5O_7 \cdot 5H_2O$, 2.5 g; $(NH_4)_2SO_4$, 3.3 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; KH_2PO_4, 0.10 g; K_2HPO_4, 0.10 g; $CaCO_3$, 1.00 g; $MnSO_4 \cdot 4H_2O$, 0.01 g; $ZnSO_4 \cdot 7H_2O$, 0.04 g; $K_2Cr_2O_7$, 0.016 mg; and CH_3COOH, 0.40 ml.

The major changes in medium throughout this developmental study reflect the effect of the phosphate-citrate-carbonate ratio. Table 2 gives the medium variations and the yields produced by culture UV8 in shaker flasks. The broth potencies obtained with this isolate in the base synthetic medium A averaged 150 μg per ml. Alterations in the phosphate concentration raised yields to an average of 290 μg per ml in medium B. The substitution of citric acid and ammonium hydroxide for sodium citrate with the citrate radical supplied at a considerably higher level resulted in an average potency of 545 μg per ml; this modification has been coded medium C. Counteracting the high acidity with increased calcium carbonate rather than ammonium hydroxide brought about a further increase in antibiotic activity. In this variation, which was coded medium D, yields were raised to an average of 940 μg/ml. A further increase in the concentration of calcium carbonate to an optimum level of 11 g per L (medium E) resulted in an additional response, and an average antibiotic broth potency of 1350 μg per ml was obtained. With culture UV184 an average yield of 2150 μg per ml was produced in this medium.

Pilot Tank Fermentations

Pilot tank fermentations paralleled those carried out in shaker flasks, and tank yields were raised from 55 μg per ml to 1235 μg per ml during the course of this investigation. Under standard tank operating conditions, which were used throughout this study, 15 gallons of medium were batched in a 25-gallon stainless steel fermentor and sterilized for 25 min at 121 C. After cooling to the operating temperature of 30 C, the batch was seeded with 24-hr shaker inoculum in an amount equal to 3 per cent of the batch volume. Agitation and an air flow of 1.5 volumes of air per volume of liquid per min were provided, and foaming was controlled by the addition of octadecanol in lard oil.

Culture UV8 gave a broth potency of 55 μg per ml after a fermentation period of 64 hr in the base synthetic medium A. In this fermentation, pH levels were highly acidic. In medium B, with changes in phosphate concentration, there was a significant increase in antibiotic activity associated with a slight elevation of the pH level; a yield of 120 μg per ml was obtained in 64 hr in this medium with culture UV8. Marked increases in broth potency and fermentation pH were evidenced in tank fermentors when the high citric acid-ammonia medium C was run; a yield of 525 μg per ml was produced in 90 hr by this culture in a typical fermentation with this medium. High calcium carbonate concentrations (10 g per L) were also effective with the high

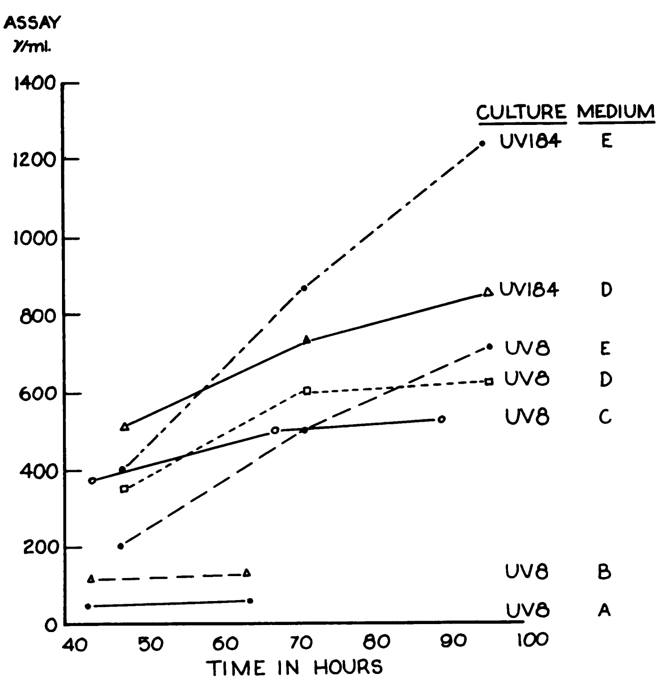

Figure 2. Effect of medium composition and strain upon tetracycline yield in tank fermentations with *Streptomyces aureofaciens.*

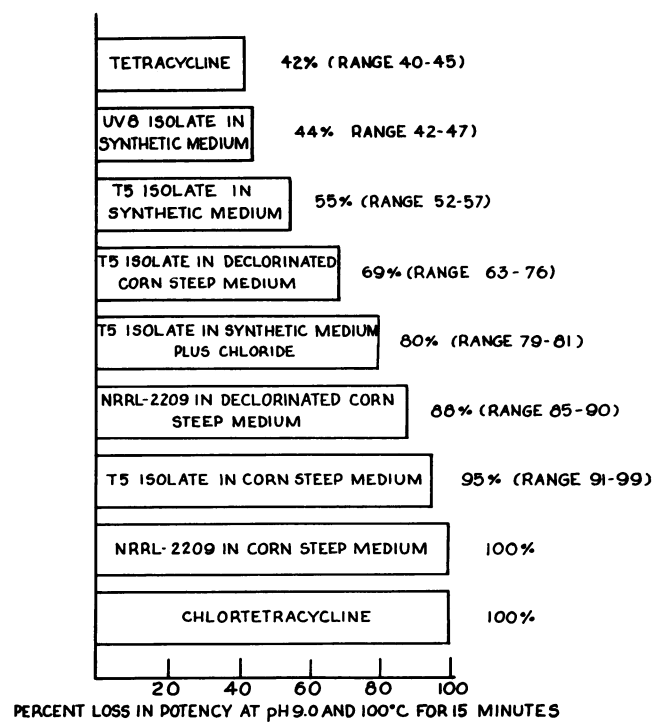

Figure 3. Stability studies

citric acid-ammonium sulfate medium. A yield of 625 μg per ml was obtained in 92 hr with culture UV8 in this modification (medium D). When the level of calcium carbonate was further increased to 11 g per L (medium E), an additional response was obtained and broth potencies were raised to 705 μg per ml in 96 hr with this culture.

Culture UV184 was tested in medium D, and 855 μg per ml tetracycline was produced in 96 hr. This culture also responded to an increase in the concentration of calcium carbonate from 10 to 11 g per L, and a broth potency of 1235 μg per ml was obtained in medium E with culture UV184 in a typical 96-hr fermentation. Figures 1 and 2 show the pH levels and yields obtained in these tank fermentations with the various media.

Broth Stability

Broth stabilities at pH 9.0 and 100 C for 15 min showed clear differentiation between chlortetracycline and tetracycline; chlortetracycline was completely inactivated and tetracycline gave 40 to 50 per cent inactivation. The stability of the antibiotic activity produced by *S. aureofaciens* cultures under different fermentation conditions was investigated and found to vary with the presence of chloride and with the individual strain. Figure 3 shows the increasing stability encountered as the fermentation was shifted from the corn steep medium of Duggar (1949) to synthetic me dium A plus chloride, to dechlorinated corn steep medium, and to synthetic medium A; at the opposite ends of this graph will be found chlortetracycline and tetracycline.

Discussion

It will be noted in table 1 that the frequency distribution of tetracycline production with the original isolate does not follow the normal distribution pattern but exhibits a marked skewness on the low side of the curve. This skewed portion is attributable to a specific morphological characteristic (that is, pale coloration) which could be readily detected and avoided. Isolates from culture T5, a superior strain selected from the other end of the curve, gave a normal distribution spread. In order to distort this curve and subsequently to obtain superior strains, mutagenic agents were applied. Since other workers including Van Dyke and De Somer (1952) and Katagiri (1954) have reported increased yields of chlortetracycline with *S. aureofaciens* when various mutagens were used, greater yields of tetracycline with *S. aureofaciens* would be expected under similar treatment.

Tetracycline fermentations in synthetic media with *S. aureofaciens* are quite similar to those reported by Van Dyke and De Somer (1952) and Biffi *et al.* (1954) for chlortetracycline. The first stage is characterized by rapid mycelial growth and the second by active antibiotic production. Since ammonium salts were used in our fermentation media, selective screening led to the development of strains which required ammonia nitrogen for optimum production of tetracycline. Various ammonium salts, ammonium hydroxide, and liquid ammonia all gave satisfactory yields.

The phosphate-citrate-carbonate ratio was found to be a highly critical one, and a study of these interrelationships raised yields in shaker flasks with culture UV8 from an average of 150 μg per ml to 1350 μg per ml. The high calcium carbonate-citric acid medium permits the calcium ion to act as a sequestering agent for tetracycline in the same manner as Niedercorn (1952) disclosed the sequestering of chlortetracycline. This medium also results in a preferred fermentation pH range which is in sharp contrast to the highly acidic pH values encountered in the base synthetic medium A. In tank fermentations this pH-assay relationship and the sequestering effect of the calcium ion are graphically illustrated in figures 1 and 2. Fermentation yields with culture UV8 were raised from 55 μg per ml in the base medium A, which results in a strongly acidic fermentation, to 525 μg per ml in the high citric acid-ammonia medium C which permits fermentation in the preferred pH range. The sequestering effect of calcium carbonate becomes apparent as the yields are further raised with this culture to an average of 705 μg per ml in medium E although the pH range during the period of active antibiotic production remains essentially unchanged.

Acknowledgment

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Summary

Details of the fermentation of tetracycline in a low chloride synthetic medium with *Streptomyces aureofaciens* have been presented along with a summary of a culture screening program following X-ray and ultraviolet irradiation.

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